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Arsenic Thiolation and the Role of Sulfate-Reducing Bacteria from the Human Intestinal Tract

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Abstract

Background: Arsenic toxicity is primarily based on its chemical speciation. While inorganic and methylated arsenic species are well characterized in terms of metabolism and formation in the human body, the origin of thiolated methylarsenicals is still unclear.

Objectives: Here, we demonstrate that sulfate reducing bacteria (SRB) from the human gut are actively involved in the thiolation of monomethylarsonic acid (MMA^V).

Methods: Human fecal and colon microbiota were incubated in batch and in a dynamic gut simulator with a dose of 0.5 mg MMA^V in the absence or presence of sodium molybdate, an SRB inhibitor. We monitored the conversion of MMA^V into monomethyl monothioarsonate ($MMMTA^V$) and other As species by HPLC-ICP-MS analysis. The SRB activity was monitored measuring the H_2S production. Molecular analysis was carried out to determine the dominant species of SRB responsible for arsenic thiolation.

Results: In the absence of sodium molybdate, the SRB activity – primarily derived from *Desulfovibrio desulfuricans* piger – was specifically and proportionally correlated ($p<0.01$) to MMA^V conversion into $MMMTA^V$. Inactivating the SRB with molybdate did not result in MMA^V thiolation but interestingly, we showed the microbiota from a dynamic gut simulator were capable of demethylating 4% of the incubated MMA^V into arsenous acid (iAs^{III}), the trivalent and more toxic form of arsenic acid (iAs^V).

Conclusion: To our knowledge, this is the first time that SRB from human gastrointestinal origin, through their ability to produce H_2S, are found to be necessary and sufficient to induce arsenic thiolation. The toxicological consequences of this microbial As speciation change are not yet clear. However, given the efficient epithelial absorption of thiolated methylarsenicals, we

conclude that the gut microbiome, and SRB activity in particular, should be incorporated into toxicokinetic analysis following As exposure.

Introduction

Arsenic (As), particularly inorganic iAs^V, is a ubiquitous contaminant, non-threshold class 1 carcinogen (Cantor and Lubin 2007; Mandal and Suzuki 2002). Global impacts of geogenic As increases the risk for elevated As exposure by consumption of contaminated drinking water and food (Francesconi 2010; Sun et al. 2012). While orally ingested and bioavailable As was previously thought to be biotransformed in the liver (Watanabe and Hirano 2012), literature also suggests As to be converted presystemically during gastrointestinal transit (Kubachka et al. 2009a; Rowland and Davies 1981; Van de Wiele et al. 2010). Presystemic arsenic metabolism is defined as the occurrence of As speciation changes due to physicochemical, enzymatic or microbial metabolic processes in the gut, prior to intestinal absorption and eventual bioavailability. Given the fact that As toxicity is primarily determined by its speciation, it is warranted to incorporate presystemic speciation changes in the risk evaluation process.

Analyses of human urine upon iAs exposure revealed sulfur-containing arsenic metabolites such as monomethylmonothioarsonic acid ($MMMTA^V$) and dimethylmonothioarsinic acid ($DMMTA^V$) (Hansen et al. 2004; Raml et al. 2007). Sulfur-containing arsenicals have also been detected in the urine and feces of experimental animals (Conklin et al. 2006; Kubachka et al. 2009b), in water (Fisher et al. 2007) and vegetables (Yathavakilla et al. 2008). In addition, thioarsenicals have been produced within the headspace of a reaction tube that contained a human fecal slurry and arsenate (Diaz-Bone et al. 2009). Furthermore, upon *in vitro* digestion of iAs^V under gastric conditions and human colon microbiota, significant arsenic thiolation takes place (Van de Wiele et al. 2010). More recently, it was shown that arsenate can be converted into methyl- and thioarsenicals by anaerobic microbiota of mouse cecum (Pinyayev et al. 2011).

However the microbial mechanism of thioarsenical formation is not well understood. Moreover, the toxicity profiles is currently under discussion (Dopp et al. 2010).

Given the importance of sulfate reduction by sulfate reducing bacteria (SRB) in the human colon (Ley et al. 2006; Marchesi 2011), previous studies hypothesize that the SRB community in the gut may play an important role in the thiolation of arsenicals (Conklin et al. 2006; Van de Wiele et al. 2010). In the present study we investigated to what extent thiolation of methylarsonic acid relies on the presence and metabolic activity of SRB from the human gut. Our findings suggest an active involvement of sulfate reducing activity towards the gastrointestinal formation of thiolated methylarsenicals.

Material and Methods

Chemicals, media, microbial cultures

Degassed and ultrapure 18 mΩ water (DDI; Millipore, Bedford, MA, USA) was used to prepare the chromatographic mobile phase and the standard stock solutions. American Chemical Society grade ammonium nitrate and ammonium dihydrogen phosphate (Fisher Scientific, Pittsburgh, PA, USA) and technical-grade EDTA, tetrasodium salt dehydrate (Fisher Scientific, Fair Lawn, NJ, USA) were used in the chromatographic mobile phase. Certified stock solutions of MMA^V and sodium arsenate ($Na_2HAsO_4{\cdot}7H2O$), sodium molybdate ($Na_2MoO_4{\cdot}2H_2O$) were purchased from Chem Service (West Chester, PA) and Sigma-Aldrich (St. Louis, MO, USA). Molybdate is not considered as a bactericidal agent. It rather is a bacteriostatic agent: this compound merely inhibits the metabolic activity, limiting SRB in their growth capability and H_2S production ability. MMA^V and iAs^V stock solutions were prepared in deionized water at 0.1 g $As.L^{-1}$ and stored at -4^oC.

$MMMTA^V$ synthesis was obtained with a mixture of MMA^V and H_2S solutions. In a 1 mL glass vial, 900 µL of a 40 µg $As.mL^{-1}$ MMA solution and 100 µL of a saturated H_2S solution were combined. The mixture was left overnight on a mechanical shaker for thorough mixing of the solutions. Progress of the reaction was verified by LC-ICP-MS. Molecular identity of the product was checked by LC-LTQ-XL-MS and MS/MS (Alava et al. 2012a). The MMA^V and H_2S solution were made according to (Alava et al. 2012a). Briefly, a 40 µg $MMA^V.mL^{-1}$ solution was prepared by combining 60 µL of a 1850 µg $MMA^V.mL^{-1}$ solution and 2.94 mL of 10% v/v formic acid solution. Preparation of saturated H_2S solution was conducted in a 100 mL RB flask. One gram of iron(II)sulfide (Harshaw Scientific, Cleveland, OH) was supplemented with 2 mL of HCl (Fisher Scientific) along with 4 mL of DDI were added. The mixture started to bubble instantly, releasing H_2S gas. The H_2S was bubbled into 15 mL of DDI water until the effervescence in the round bottom flask subsided. This DDI water was used as saturated H_2S solution.

The Simulator of the Human Intestinal Microbial Ecosystem (SHIME) is a dynamic multi-compartment simulator of the human gastrointestinal tract, mimicking the digestive processes in stomach, small intestine and ascending, transverse and descending colon. The model has been validated against human *in vivo* conditions both in terms of gut microbial composition and metabolic activity (short chain fatty acid profile) (Molly et al. 1994; Possemiers et al. 2006). The nutritional medium for the dynamic gut model Simulator of the Human Intestinal Microbial Ecosystem (SHIME) was made as described before (Boever et al. 2000), which enabled the microbial community of the different colon compartments to adapt to the nutritional and physicochemical conditions that prevail in the ascending, transverse and descending colon,

respectively. Briefly, one liter of SHIME medium contains arabinogalactan 1g, pectine 2g, xylan 1g, starch 3g, glucose 0.4g, yeast extract 3g, pepton 1g, mucin 4g, cystein 0.5g at pH 7.

Postgate medium C (Grossman and Postgate 1953) was used to enrich SRB. It consisted of 4.5g Na_2SO_4, 0.5g KH_2PO_4, 0.06g $MgSO_4 \cdot 7H_2O$, 1.0g NH_4Cl, 0.06g $CaCl_2 \cdot 2H_2O$, 1g yeast extract, 0.1g ascorbic acid, 0.004g $FeSO_4 \cdot 7H_2O$, 6g sodium lactate, 0.3g sodium citrate at pH 7.5. Modified Postgate medium C with different sulfate concentrations was obtained using a 4-fold dilution series in the concentration range 0.007, 0.032, 0.125 and 0.5M of Na_2SO_4.

A pure culture of *Desulfovibrio desulfuricans* LMG 7529 was purchased from the Belgian Co-Ordinated Collections Of Micro-Organisms and was grown in the recommended medium 104 from (BCCM-LMG, Belgium). This strain is equal to ATCC 29577. *D. desulfuricans* is still considered *D. piger* (Castro et al. 2000).

Batch incubations of non-enriched, enriched and pure cultures

A first set of experiments checked to what extent the *in vitro* cultured gut microbiota from the human inoculum was capable of performing iAs^V biotransformation in a similar manner to some of our previous findings (Alava et al. 2012a; Van de Wiele et al. 2010). Briefly, 2 mL of descending colon suspension from SHIME (*vide infra*) was anaerobically incubated with 30 µg $iAs^V.L^{-1}$ for 48h and then analyzed for its As speciation profile with HPLC-ICP-MS as detailed in Van de Wiele et al. (2010).

The second set of experiments was more specifically targeted at demonstrating the potential of gut microbiota and SRB to thiolate MMA^V. Two mL of SRB non-enriched descending colon suspension from the SHIME were anaerobically incubated for 48 h with 0.5 $mg.L^{-1}$ MMA^V. To favor SRB enrichment, 2 mL was sampled from SHIME descending colon suspension and

anaerobically incubated for 48h with 0.5 mg.L^{-1} MMA^V in 18 mL Postgate medium C. The contribution of a reference sulfate reducing strain towards MMA^V (0.5 mg.L^{-1}) thiolation was assessed by incubation of 2 mL of a pure culture of *Desulfovibrio desulfuricans (piger)* in 18 mL of culture medium 104 (BCCM-LMG, Belgium).

A third set of experiments tested the interindividual variability in MMA^V thiolation by human fecal microbiota. Fecal microbiota from 7 different human individuals with no history of antibiotic treatment in the 6 months prior to the study (De Weirdt et al. 2010) and descending colon samples from 3 different SHIMEs were separately incubated with 0.5 mg.L^{-1} MMA^V in Postgate medium C.

All incubation experiments with SRB enriched and non-enriched SHIME descending colon samples, human fecal microbiota and with *Desulfovibrio desulfuricans* were performed in the absence or presence of sodium molybdate (20mM), a specific inhibitor for SRB. In addition, heat-sterilized (120 °C) incubations were used as abiotic control. Incubations were performed under anaerobic conditions by capping the serum bottles with butyl rubber stoppers that are impervious to O_2 and subsequently flushing these recipients with N_2 gas for 25 min (over and under-pressure). Cultures were then incubated at 37°C on a rotary shaker (180 rpm) for 48 hr. Aliquots of 2 mL/analysis were collected at four time points 0, 6, 24 and 48 hours to monitor SRB activity, the As speciation changes as well as molecular analysis of the microbiota. This study has been approved by Ghent University's ethical committee and has been registered by Belgian authorities with number B670201214538.

Continuous incubations in a dynamic gut simulator (SHIME)

While the former batch experiments were conducted under SRB favoring conditions, a SHIME run was carried out to verify whether MMA^V speciation changes, particularly thiolation, also

occurred under more representative conditions for the human gut that do not favor SRB. Moreover, the SHIME reactor also allows addressing colon region specific differences in the MMA^V thiolation potential.

The treatment consisted of a daily supplementation of 0.5 mg $MMA^V.L^{-1}$ during four days. 20mM of sodium molybdate (SRB inhibitor) was added on the third and fourth day of the SHIME run to inhibit SRB activity. Aliquots of 2 mL/analysis were collected from the ascending, transverse and descending colon in order to monitor the conversion of MMA^V as well as SRB activity.

SRB activity analysis and sample preparation for speciation analysis.

SRB activity was monitored measuring H_2S production using an analytical kit for detection of sulfide in an automated spectrophotometer (Hach, Loveland, CO, USA) in dilutions 1:1, 1:2 with anoxic water. To preserve the samples for further As speciation analysis, all samples were flash frozen with liquid nitrogen upon incubation and subsequently stored at –80°C. Before analysis with high-performance liquid chromatography coupled with inductively coupled plasma mass spectrometry (HPLC-ICP-MS), samples were thawed and dissolved with $(NH_4)_2CO_3$ at (20 mM, pH 9.0) to minimize sulfur–oxygen exchange while awaiting analysis (Conklin et al. 2008). Upon complete thawing, the sample was vortexed and centrifuged for 10 min at 10,400 *rcf* with an Eppendorf 5810R centrifuge (Brinkman Instruments, Westburg, NY, USA) to separate soluble As species from insoluble As (sorbed to microbial biomass etc.). The supernatant was filtered through a Millex-LCR 0.45 μm filter (Millipore) with a Luer-Lok 10 mL syringe (BD, Franklin Lakes, NJ, USA).

As speciation analysis by HPLC-ICP-MS

As speciation changes and especially the conversion of MMA^V into monomethyl monothioarsenate ($MMMTA^V$) and arsenous acid (As^{III}) were monitored with HPLC-ICP-MS matching the retention time and by comparing fragmentation pattern of prepared $MMMTA^V$ on ESI-MS/MS with already published conditions (Van de Wiele et al., 2010) using the limits of detection and quantification for the different As species indicated in Supplemental Material, Table S1. Briefly 2 mL of the supernatant of incubated samples were filtered using 0.45 µm syringe-type PVDF membrane filter and the filtrate was diluted into 25 mL using double distilled deionized water. This filtrate was analysed for total arsenic content using ICP-MS. The same filtrate was used for speciation analysis using HPLC and optimized instrumental parameters for ICP-MS (Perkin Elmer, Sunnyvale, CA, USA). Filtrates were diluted with the mobile phase and injected into the HPLC. The sum of the arsenic species in the filtrate observed chromatographically was considered the bioaccessible fraction. We measured total As concentration in the digest filtrates using ICP optical emission spectroscopy (ICP-OES) according to (Alava et al. 2012a; Alava et al. 2012b). The applicable detection limit was 0.5 $\mu g.L^{-1}$.

Molecular analysis

PCR-DGGE was performed to obtain a general profile of the microbial community, qPCR was used to quantify the SRB, while a clone library was made to identify the most dominant SRB species in the SRB non-enriched and SRB enriched incubation experiments. DNA extraction was carried out using the UltraClean® DNA Isolation Kit following manufacturers instructions (Mo Bio Laboratories, Inc). PCR-DGGE of the 16S rRNA genes for all bacteria were amplified by PCR using the Taq-polymerase kit (Fermentas) with general bacterial primers P338F and P518R

and a GC-clamp of 40 bp on the forward primer (Muyzer et al. 1993). DGGE was performed using the Bio-Rad D gene system (Bio-Rad, Hercules, CA). Clustering was based on the densitometric curves according to Pearson correlation using Bionumerics program. For the clone library see Supplemental Material. Briefly, the PCR amplification of 16S rRNA gene fragments was carried out with the universal primers 63F and 1378R and cloned into the pCR®-TOPO® Vector of the TOPO TA cloning kit (Invitrogen, California). The qPCR specific for SRB's target gene (dissimilatory sulfite reductase beta-subunit, dsrB) key enzyme in dissimilatory sulfate reduction and phylogenetic marker for identification of SRBs was carried out as (Vermeiren 2011) adapted from (Spence et al. 2008) (See Supplemental Material).

Statistics and sequences

Batch incubation experiments of more than four groups were conducted in triplicate and SHIME run in duplicates. All data were analyzed using SigmaPlot Version 12.0. A one-way ANOVA test was performed to investigate intergroup differences. Two case groups were covered by a t-test. Statistical differences for ANOVA and t-tests were significant if $p \leq 0.05$ and highly significant if $p \leq 0.01$. The nucleotide sequences data of the clone library have been submitted to a public database EMBL-EBI (Accession Number: HG531812 to HG531931).

Results

Batch incubation experiments with SHIME descending colon microbiota showed that the human gut microbiota are capable of actively metabolizing iAs^{V} (30 μg=100%) (Table 1). Importantly 7% $MMMTA^{V}$ formation was observed upon 48-hr of incubation. In addition iAs^{V} was reduced to As^{III} (18%) and further transformation towards Monomethyl arsonous acid (MMA^{III}) (6.6%), MMA^{V} (3.2%) and DMA^{V} (54%) was noted. These data demonstrate that the *in vitro* cultured

microbial community from the human inoculum in these experiments had the potency to actively metabolize iAs^V (Table 1).

Subsequently, we investigated to what extent sulfate reducing bacteria (SRB) are involved in the thiolation of MMA^V towards $MMMTA^V$. The batch incubations showed that SRB can be enriched with Postgate medium C and can be inhibited by addition of Na-molybdate and this was reflected in the sulfide production potential (Figure 1A). Molybdate is a well-known inhibitor of ATP-sulfurylase, thereby inhibiting SRB to make sulfide, but also limiting these SRB to generate energy: growth will therefore be affected (Figure S1). Furthermore, we found that descending colon microbiota under SRB-enriched conditions produced significantly more $MMMTA^V$ (28 $\mu g.L^{-1}$) than under non-enriched conditions (15 $\mu g.L^{-1}$) ($P<0.01$). In contrast, no MMA^V to $MMMTA^V$ conversion was observed when the SRB-inhibitor Na-molybdate was supplemented (Figure 1B). In addition, both the incubations with SRB-enriched cultures and non-enriched cultures displayed a positive correlation between the formation of $MMMTA^V$ and production of H_2S ($R^2=0.978$ and $R^2=0.992$, respectively). Finally, no speciation changes were observed in the abiotic control where heat-sterilized colon microbiota were incubated. However, the fraction of MMA^V remaining in the supernatant declined because of sorption to the dead organic biomass (see Supplemental Material, Table S3). This demonstrates the necessity of sulfate reducing activity to contribute to the thiolation process while inactivation through a specific inhibitor or heat-sterilization removes the thiolation ability. It must be noted that the presence of H_2S as such suffices to chemically produce $MMMTA^V$ from MMA^V (see M&M Chemicals, media, microbial cultures). Hence the As thiolation in the gut can be considered a chemical process that requires a biological trigger, i.e. sulfide production by metabolically active SRB.

To identify the dominant microbial species in SRB enriched cultures that was responsible for the thiolation of MMA^V, molecular analysis was carried out from the SRB enriched and non-enriched cultures. Analysis from the clone library revealed a dominant sequence over time (from 18% to 63% at 6hrs to 48hrs respectively) with 99% of similarity to *D. desulfuricans (piger*) (Figure 2A). PCR-DGGE shown a SRB predominant band over the time observed in the enriched cultures (Figure S2). Quantitative analysis with qPCR further confirmed the increasing abundance of SRB in the enriched cultures (Figure 2B). In addition, illumina sequencing of non-enriched fecal incubations showed that *D. desulfuricans (piger)* is the most dominant SRB present in human gut (data not shown). Incubations of pure cultures of *D. desulfuricans (piger)* displayed a similar sulfate reducing activity and As thiolation ability as with the SRB enriched colon microbiota (Figure S4).

Using SHIME as a dynamic simulator of the human gut (Figure S5), we then investigated whether thiolation of 0.5 $mg.L^{-1}$ MMA^V is colon region specific under more representative conditions for the gastrointestinal tract (Figure 3). MMA^V thiolation was observed in the SHIME with $MMMTA^V$ formation primarily taking place in the ascending and transverse colon compartments at a rate of > 30 $\mu g.\ L^{-1}$ .day (Figure 3A, 3B). This resulted in high amounts of $MMMTA^V$ (more than 35 $\mu g.\ L^{-1}$) in the ascending and transverse colon vessels, whereas only a minor amount was observed in the descending colon (Figure 3C). $MMMTA^V$ formation took place within the first 10 hours upon supplementation of MMA^V (Figure 3). Adding sodium molybdate on the third and fourth day of the SHIME run to eliminate SRB activity, did not result in a drop of MMA^V conversion. Instead of $MMMTA^V$ formation, demethylation of MMA^V took place towards iAs^{III}, a process, which primarily took place in the distal colon regions (Figure

3C). For SHIME reactor distal colon regions and a scheme of As speciation see Supplemental Material, Figure S5, S6.

Finally, interindividual variability was observed in the sulfate reducing activity and MMA^V thiolation between different human fecal inocula (Figure 4). The fecal microbiota from individuals A and C displayed much higher levels of H_2S (>15 $mg.L^{-1}$ H_2S) in comparison with the fecal microbiota from the other individuals (Figure 4A). This H_2S production from fecal microbiota A and C corresponded with a pronounced production of $MMMTA^V$ (more than 20 $\mu g.L^{-1}$) (Figure 4B). In contrast, for those fecal microbiota that displayed low SRB activity (around 5 $mg.L^{-1}$ of H_2S), only a limited amount of $MMMTA^V$ was formed over time (around 4.5 $\mu g.L^{-1}$ at 24 and 48-hr). Moreover, the fecal microbial inoculum G displayed the lowest H_2S production (<2.5 $mg.L^{-1}$) and no formation of $MMMTA^V$. Overall, $MMMTA^V$ formation and H_2S production by fecal microbiota from the different individuals were strongly correlated to one another (R^2=0.994) after 48-hr.

Discussion

The present study demonstrates that human colon microorganisms have the potency of presystemic As metabolism, similar to results obtained before with rodent (Conklin et al. 2006; Kubachka et al. 2009a) and human gut microbiota (Van de Wiele et al. 2010). Moreover, this is the first study that shows the active involvement of sulfate reducing bacteria (SRB) from human origin to contribute to the thiolation of MMA^V into $MMMTA^V$. We observed this process both under SRB enriched and non-enriched *in vitro* cultures of the descending colon, of human fecal microbiota and of pure SRB isolates, as well as under more representative conditions for the human gut in the SHIME, a dynamic gut simulator. The active contribution of SRB was demonstrated by the high correlation between H_2S production and $MMMTA^V$ formation and by

the lack of $MMMTA^V$ formation and H_2S production in the presence of molybdate, an SRB inhibitor. Moreover, we have indicated that *D. desulfuricans (piger)* may be the principal microbe contributing to the arsenic thiolation process. While the metabolic activity of SRB has been well studied and even implicated in the methylation process of mercury (Gilmour et al. 2011), the role of metabolically active SRB and particularly *D. desulfuricans (piger)* towards As-thiolation is a new finding.

These observations parallel those studies showing $MMMTA^V$ formation upon incubation of iAs^V with human colon microbiota (Van de Wiele et al. 2010) or the formation of headspace thio-arsenicals when a human fecal slurry was incubated with arsenate (Diaz-Bone et al. 2009). Previous observations have shown that mouse cecal microbiota can trigger the formation of thioarsenosugars upon the incubation with arsenosugars (Conklin et al. 2006) as well as the production of methylated thioarsenicals from DMA^V by rat intestinal microbiota (Yoshida et al. 2001) and DMA^V conversion into trimethylarsine sulfide by mouse ceca (Kubachka et al. 2009a). Therefore, the presence of SRB in both the human and different animal gastrointestinal environments may be considered as an important factor in the arsenic thiolation process, and, when considering environmental presence of SRB, to also impact biogeochemical cycle of sulfur and arsenic (Muyzer and Stams 2008). Yet, it must be noted that the arsenic thiolation rather concerns a chemical reaction that is biologically induced by metabolically active SRB. This view is supported by the possibility to chemically produce $MMMTA^V$ by the reaction of MMA^V with a saturated H_2S solution. This corresponds with previous hypotheses that presence of sulfide is sufficient to obtain interconversion between oxide and sulfide forms of MMA^V, DMA^V and trimethyl arsine oxide (Conklin et al. 2006).

While the importance of As thiolation by endogenous SRB can be derived from the present dataset, the results also demonstrate that the thiolation does not take place at the same rate among the entire gastrointestinal tract. As thiolation appears to be colon region specific in this study: thiolation primarily took place in ascending and transverse colon. This observation is strongly supported by the fact that in the *in vivo* human colon, the SRB are more abundant in ascending and transverse colon, while homo-acetogens (which compete for reducing equivalents) are more abundant than SRB in the descending colon (Nava et al. 2012).

Additionally, the inactivation of SRB activity by Na-molybdate in the SHIME colon compartments resulted in the demethylation of MMA^{V} towards iAs^{III} by descending colon microbiota. While demethylation of MMA^{V} was previously reported for soil microbial communities (Yoshinaga et al. 2011), this is to our knowledge the first study that demonstrates As demethylation by human colon microorganisms. These findings are of toxicological concern. One the one hand, MMA^{V} demethylation is rather unexpected as iAs^{III} is more toxic than MMA^{V} (Naranmandura et al. 2011; Van de Wiele et al. 2010). On the other hand, the strongly reducing conditions that prevail in the SHIME colon compartments (-200mV to -250mV) may lead to the reduction of MMA^{V} towards its trivalent analogue, MMA^{III}. The ability of this As species to generate highly reactive oxygen species and induce DNA damage makes it an order of magnitude more toxic than iAs^{III} (Naranmandura et al. 2011). Although MMA^{III} was not detected under the dynamic incubation conditions from the SHIME, its production as intermediate is likely, as was also demonstrated by the finding of considerable amounts of MMA^{III} upon static incubation of iAs^{V} (Table 1). Supported by reports that $MMMTA^{V}$ is several orders of magnitude less toxic than iAs^{III} and even less toxic than iAs^{V}, we therefore consider intestinal MMA^{V} thiolation to be a detoxification reaction, however it still needs further investigation.

Thiolated arsenicals display a highly variable toxicity profile. The monothiolated form of DMA^V, $DMMTA^V$ – often found in the urine of iAs exposed individuals (Heitland and Köster 2008; Raml et al. 2007) – is one of the most toxic As species known, comparable to DMA^{III}, while its dithiolated analogue, $DMDTA^V$ is almost harmless (Naranmandura et al. 2011). Whether SRB also contribute to the formation of $DMMTA^V$, just as they do for $MMMTA^V$, still needs to be resolved.

Finally, As thiolation was characterized by a large interindividual variability. Again, the ability for a fecal microbiome to produce $MMMTA^V$ correlated with the levels of H_2S, further supporting the role for SRB to lie at the basis of the thiolation process. Despite the enterotypes (Arumugam et al. 2011) the human gut microbiome is known for its high interindividual variability and this could also be reflected upon the variable abundance of SRB in the colon lumen or colon mucosal surfaces (Nava et al. 2012). While host genetic factors have been reported to contribute to the interindividual variability in As toxicity (Hernandez and Marcos 2008), we propose that the gut microbiome must be incorporated as a factor that contributes to this variability.

Conclusion

From the present research we can conclude that gut microbiota from human origin can extensively metabolize As with sulfate reducing bacteria being necessary and sufficient for the biologically induced thiolation of MMA^V into $MMMTA^V$. The variability in the thiolation potency between different fecal inocula was reflected by a large interindividual variability in SRB abundance. In addition, eliminating SRB activity (H_2S production) may also result in MMA^V demethylation to iAs^{III}. While the toxicological consequences of these microbial processes are not yet clear and the interindividual variability adds an extra layer of complexity

over As toxicokinetics, our findings demonstrate the necessity to consider SRB and by extension the human gut microbiome when assessing risks from oral As exposure.

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Table 1. Metabolic potency of colon microbiota towards iAs^V speciation of a single experiment (%).

Time (h)	As^{Va}	As^{III}	MMA^V	MMA^{III}	$MMMTA^V$	DMA^V	DMA^{III}
0	100.0	-	-	-	-	-	-
5	5.1	32.1	3.9	-	-	50.1	-
8	4.9	31.2	3.7	3.7	1.2	59.8	-
24	6.7	18.1	5.6	4.7	3.4	64.5	-
48	6.1	18.2	3.2	6.6	7.0	54.3	-

[a] As^V standard at high concentration = 30 µg (100%).

Figure legends

Figure 1. Sulfate reducing activity correlate with $MMMTA^{V}$ formation. A) Sulfate reducing activity (H_2S production) and B) $MMMTA^{V}$ formation of enriched SRB (with and without Na-molybdate, the SRB inhibitor), non-enriched and abiotic (sterilized) cultures during 48 hr of incubation with MMA^{v}. ANOVA one way shown significant (*$p < 0.05$) and highly significant (**$p < 0.01$) statistical differences (grey asterisk) between the intergroup at 6 and 24 to 48 hours respectively. A two case t-test based on Non-Enriched SRB (intermediary group) shown statistical differences (black asterisks) with significant (*$p < 0.05$) at 6 and with highly significant (**$p < 0.01$) at 24 to 48 hours of Enriched SRB and controls.

Figure 2. Molecular analysis of sulfate reducing cultures. A) Clone library of 16S at genus level (OTU at 0.03%) during the enrichments of SRB culture in Postgate medium C. B) Relative number of copies normalized to SRB dsrB gene of non-enriched, enriched and fecal samples during 48-hr of incubation. Values are represented by mean ± SD, n=3.

Figure 3. Arsenic speciation of MMA^{V} into $MMMTA^{V}$ and As^{III} in the dynamical gut model SHIME. Arsenic speciation i.e. thiolation of MMA^{V} into $MMMTA^{V}$ or demethylation of MMA^{V} into As^{III}, was daily measured during four days (24, 25, 26, 27^{th}) at two time points: 10 and 16 hr. A) Arsenic speciation in ascendant colon (AC). Vertical blue arrow indicates the day of the addition of SRB inhibitor, Na-molybdate. B) Arsenic speciation in transversal colon (TC). C) Arsenic speciation in descendent colon (DC). Horizontal black arrow indicated the sequence of colon compartments of the SHIME.

Figure 4. Interindividual variability of sulfate reducing activity and thiolation in different human fecal samples. A) Sulfate reducing activity (H_2S production) and B) $MMMTA^{V}$ formation in different human fecal samples during 48 hr of incubation with MMA^{v}. Abiotic controls are represented by the heat sterilized incubation of individual A.

ANOVA one way and a two case t-test shown highly significant (**$p < 0.01$) statistical differences at 24 to 48 hours of the individual A and C compared with other groups.

Figure 1.

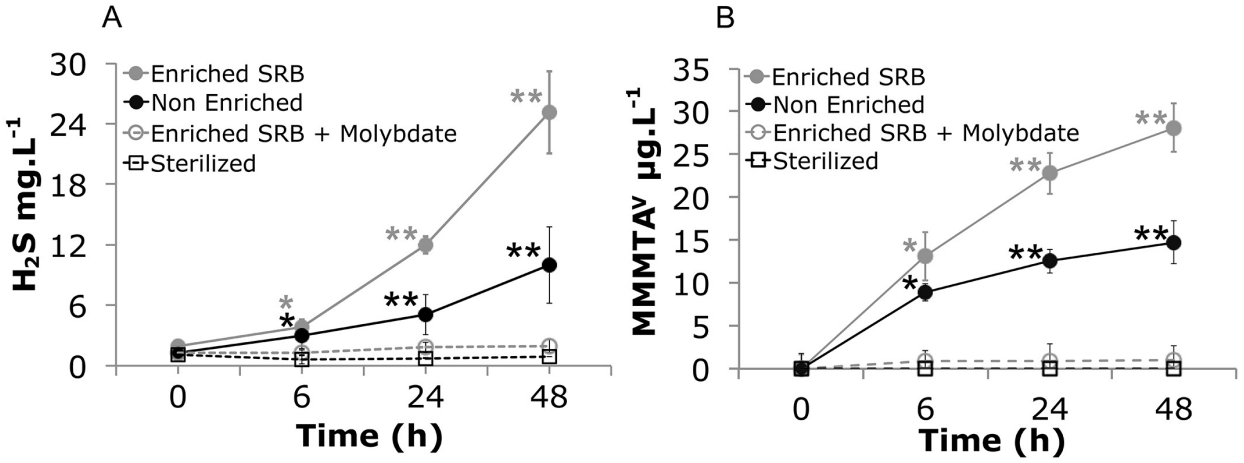
A
B
Enriched SRB
Non Enriched
Enriched SRB + Molybdate
Sterilized
H2S mg.L-1
MMMTAv µg.L-1
Time (h)
0
6
24
48
30
24
18
12
6
0
35
30
25
20
15
10
5
0
*
**

Figure 2.

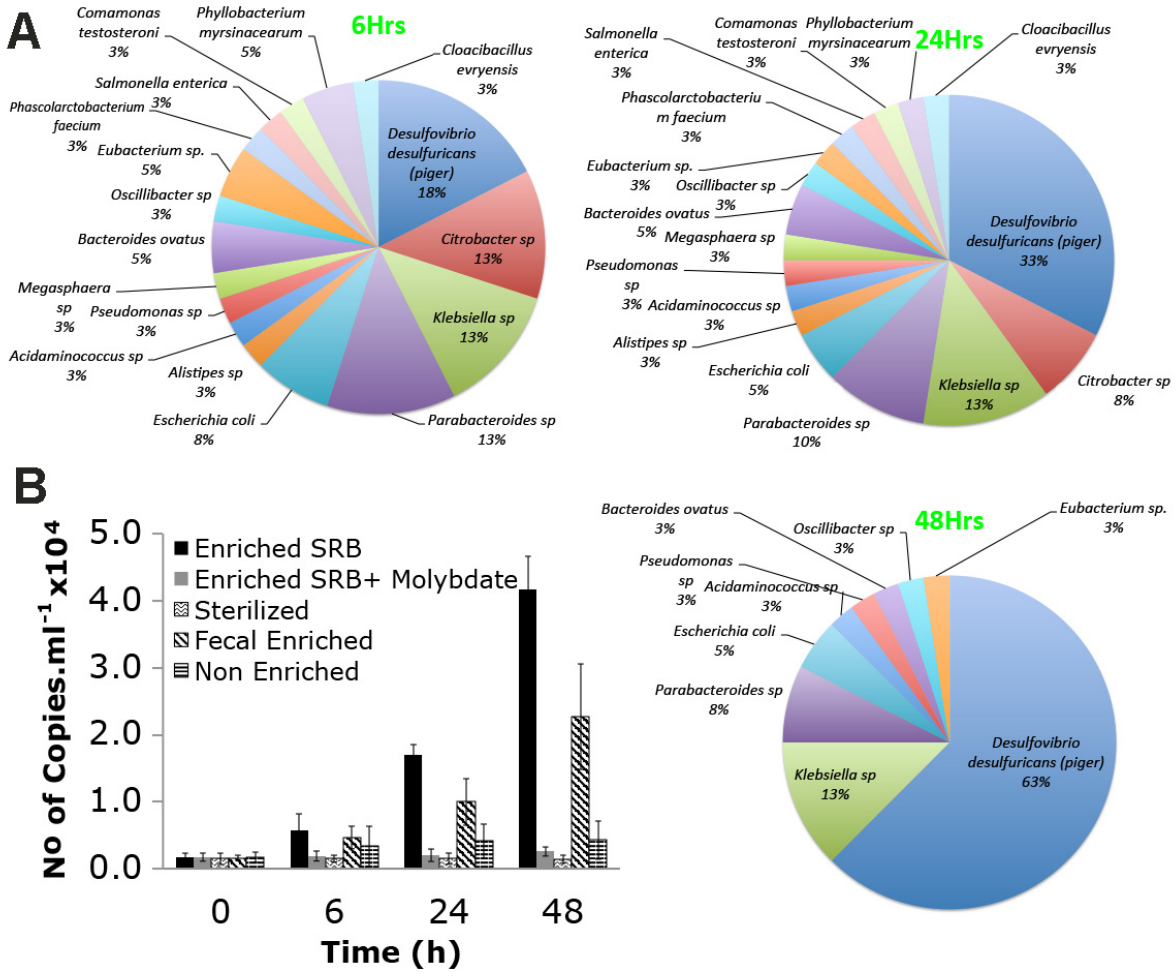
A
6Hrs
Comamonas testosteroni 3%
Phyllobacterium myrsinacearum 5%
Cloacibacillus evryensis 3%
Salmonella enterica 3%
Phascolarctobacterium faecium 3%
Eubacterium sp. 5%
Oscillibacter sp 3%
Bacteroides ovatus 5%
Megasphaera sp 3%
Pseudomonas sp 3%
Acidaminococcus sp 3%
Alistipes sp 3%
Escherichia coli 8%
Parabacteroides sp 13%
Desulfovibrio desulfuricans (piger) 18%
Citrobacter sp 13%
Klebsiella sp 13%
24Hrs
Salmonella enterica 3%
Comamonas testosteroni 3%
Phyllobacterium myrsinacearum 3%
Cloacibacillus evryensis 3%
Phascolarctobacterium faecium 3%
Eubacterium sp. 3%
Oscillibacter sp 3%
Bacteroides ovatus 5%
Megasphaera sp 3%
Pseudomonas sp 3%
Acidaminococcus sp 3%
Alistipes sp 3%
Escherichia coli 5%
Parabacteroides sp 10%
Desulfovibrio desulfuricans (piger) 33%
Klebsiella sp 13%
Citrobacter sp 8%
B
No of Copies.ml-1x10^4
5.0
4.0
3.0
2.0
1.0
0.0
Enriched SRB
Enriched SRB+ Molybdate
Sterilized
Fecal Enriched
Non Enriched
0
6
24
48
Time (h)
48Hrs
Bacteroides ovatus 3%
Oscillibacter sp 3%
Eubacterium sp. 3%
Pseudomonas sp 3%
Acidaminococcus sp 3%
Escherichia coli 5%
Parabacteroides sp 8%
Klebsiella sp 13%
Desulfovibrio desulfuricans (piger) 63%

Figure 3.

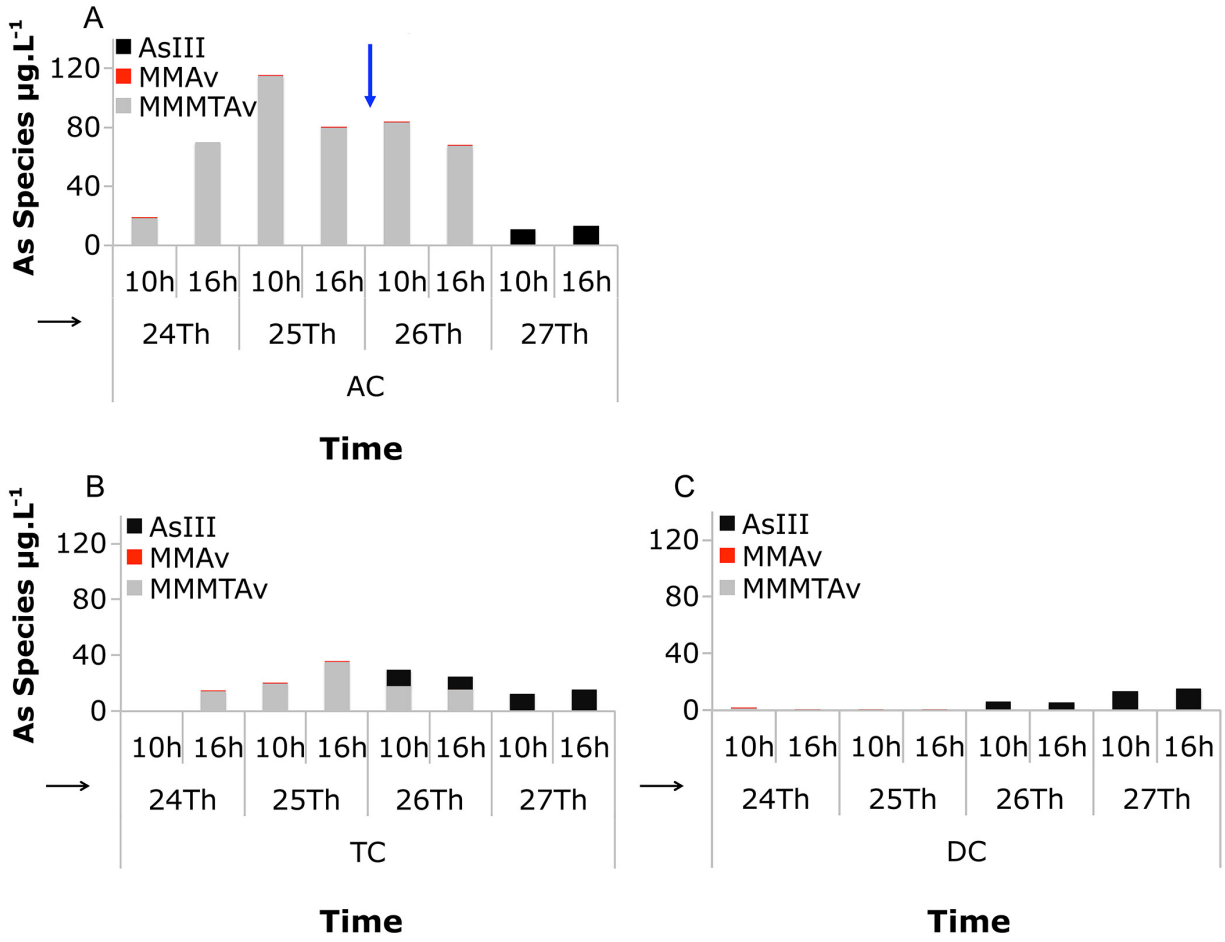
A
AsIII
MMAv
MMMTAv
As Species µg.L-1
120
80
40
0
10h 16h 10h 16h 10h 16h 10h 16h
24Th 25Th 26Th 27Th
AC
Time
B
AsIII
MMAv
MMMTAv
As Species µg.L-1
120
80
40
0
10h 16h 10h 16h 10h 16h 10h 16h
24Th 25Th 26Th 27Th
TC
Time
C
AsIII
MMAv
MMMTAv
120
80
40
0
10h 16h 10h 16h 10h 16h 10h 16h
24Th 25Th 26Th 27Th
DC
Time

Figure 4.